

A New Mitochondrial DNA Mutation at 14577 T/C Is Probably a Major Pathogenic Mutation for Maternally Inherited Type 2 Diabetes

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From a family of 16 diabetic patients with typical maternal inheritance, we investigated a 69-year-old woman with type 2 diabetes. The proband showed no major deletions in the mitochondrial DNA (mtDNA). Direct sequencing revealed 7 missense and 5 ribosomal RNA homoplasmic nucleotide substitutions when compared with the Cambridge Sequence and its recent revision. When compared with the control cybrid cells, the proband cybrid cells showed 6 nucleotide substitutions. Among these, 14577 T/C, which turned out to be 98.9% heteroplasmic, is a new missense substitution in the NADH dehydrogenase 6 gene. We also observed 2 other patients with 14577 T/C substitution from another group of 252 unrelated diabetic patients, whereas no individual from a group of 529 control subjects had 14577 T/C substitution. Furthermore, these 6 substitutions were in linkage disequilibrium. Mitochondrial respiratory chain complex I activity and O₂ consumption rates of the proband cybrid cells, which were obtained by the fusion of mtDNA-deleted (ρ^0) HeLa cells and mtDNA from the proband, showed 64.5 and 61.5% reductions, respectively, compared with control cybrid cells. The present study strongly indicates that the new mtDNA mutation at 14577 T/C is probably a major pathogenic mutation for type 2 diabetes in this family. *Diabetes* 49:1269–1272, 2000

Type 2 diabetes is often transmitted in a mode of maternal inheritance (1), and mutations in mitochondrial DNA (mtDNA) are thought to be responsible for the pathogenesis of the disease (2,3). It has been reported that mtDNA 3243 heteroplasmic mutation can be observed in about 1–1.5% of patients with type 2 diabetes (4–6). We, on the other hand, reported that 5.14% of Japanese patients ($n = 253$) with type 2 diabetes are associated with mtDNA nucleotide substitutions at 1310 C/T and/or 12026 A/G (7). To establish the role of these substitu-

tions in the pathogenesis of diabetes, it is necessary to verify impairment in mitochondrial functions due to these substitutions. Cybrid cells obtained by the fusion of mtDNA-deleted (ρ^0) HeLa cells and mtDNA from a patient allow us to evaluate mitochondrial functions due solely to mtDNA substitutions. In this study, we investigated mtDNA and mitochondrial functions using cybrid system in a patient with maternally inherited type 2 diabetes.

Southern blot analysis (Fig. 1) revealed no major mtDNA deletions in leukocytes of the proband and 3 children. Direct sequencing of the entire mtDNA, except the D-loop region, revealed 23 essentially homoplasmic nucleotide substitutions, including 11 innocent substitutions, compared with the Cambridge Sequence (8) and its recent revision (9) (Table 1). The identical substitutions were also observed in buccal mucosa, fibroblasts, and cybrid cells of the proband. Moreover, the identical substitutions were also observed in the peripheral blood of subjects III-9, III-10, IV-10, IV-11, and IV-12 (Fig. 2). However, heteroplasmic mutations at 3243, 3250, 3251, 3252, 3254, 3256, 3260, 3271, 3302, and 3303 in tRNA^{Leu(UUR)} (3) were not observed in the leukocytes and cybrid cells of the proband by direct sequencing (data not shown). In regard to 3243 mutation, we could not detect heteroplasmic mutation by *Apa* I digestion of a polymerase chain reaction (PCR) product with labeled primers (data not shown). We compared sequencing of the controls and the proband cybrid cells, and observed 6 nucleotide differences (Table 1). These substitutions, except 14577 T/C, have already been reported (<http://www.gen.emory.edu/MITOMAP>). Because the proband showed 5178 C/A substitution, which disrupts the 5176 *Alu* I site, the proband belongs to Asian mtDNA haplotype D (10). Furthermore, 14577 T/C is a new substitution and causes amino acid replacement of valine for isoleucine at codon 33 of the NADH dehydrogenase (ND) 6 gene (Table 1). Subcloning of this region revealed 1.1% (1 of 93) of T and 98.9% (92 of 93) of C at 14577. Thus, 14577 T/C mutation turned out to be 98.9% heteroplasmic.

The substitutions at 3010 G/A, 5178 C/A, and 8414 C/T were observed in >30% of both diabetic patients and control subjects, and these 3 substitutions were in linkage disequilibrium ($P < 0.0001$) (Table 2). Our observation agreed with the report by Tanaka et al. (11). There were no significant differences in the prevalences of 3206 C/T and 14979 T/C substitutions between the 2 groups, whereas the prevalence of 14577 T/C substitution in diabetic patients (3 of 253) was significantly higher than that of control subjects (0 of 529)

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FBS, fetal bovine serum; LHON, Leber hereditary optic neuropathy; mtDNA, mitochondrial DNA; ND, NADH dehydrogenase; PCR, polymerase chain reaction.

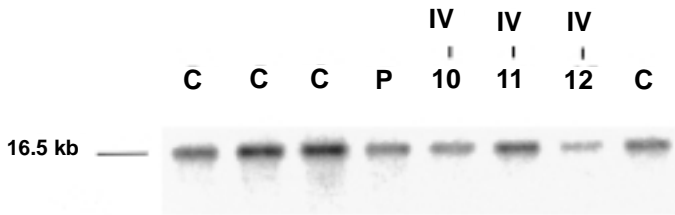


FIG. 1. Southern blot analysis. Total genomic DNA (3 µg) was digested by *PvuII* and separated by electrophoresis on 0.8% agarose gel. DNA samples were transferred to a nylon membrane and hybridized with the 15.6-kb mtDNA probe as described in RESEARCH DESIGN AND METHODS. The autoradiography was processed by Bas 2000. The other lanes correspond with the individuals shown in Fig. 2. C, control subject; P, proband.

($P = 0.0336$) (Table 2). Furthermore, those 3 patients with 14577 T/C were all associated with 5 other substitutions. Therefore, these 6 substitutions were also in linkage disequilibrium ($P < 0.0001$) (Table 2). Among the 3 patients with 14577 T/C substitution, we observed nucleotide differences at 16093 and 182 in the D-loop region compared with the Cambridge Sequence (8). Although we suppose that these 3 patients may have originated from a common ancestor, we think that these 3 patients are not directly related.

In a cybrid study, respiratory chain complex I activity and total oxygen consumption of the proband cybrid cells showed 64.5 and 61.5% reductions, respectively, whereas complex IV activity showed 17.3% reduction compared with age-matched control cybrid cells (Table 3). There was almost no difference in mtDNA contents between the controls and the proband cybrid cells (data not shown). Moreover, discrepancy of the impairments between complex I and IV activities of the proband cybrid cells also indicates that the substantial decrease in complex I activity of the proband cannot be explained by the decrease in mtDNA contents. Thus, we attributed mitochondrial impairments in the proband cybrid cells to qualitative changes of mtDNA. Among the 6 substitutions, 5178 C/A and 14577 T/C are compatible with the results of the cybrid study, because these are missense substitutions in ND 2 and ND 6 genes, respectively. However, because 5178 C/A was observed in >30% of the subjects (Table 2), we think

TABLE 1
Mitochondrial DNA homoplasmic substitutions observed in the proband

Position	Nucleotide change	Gene change	Amino acid change
750*	A/G	12S	rRNA
1438*	A/G	12S	
2707*	A/G	16S	
3010	G/A	16S	
3206	C/T	16S	
5178	C/A	ND 2	L237M
8414	C/T	ATP 8	L 17F
8860*	A/G	ATP 6	T112A
14577	T/C	ND 6	I33V
14766*	C/T	Cyt b	T7I
14979	T/C	Cyt b	I78T
15326*	A/G	Cyt b	T194A

ATP, ATP synthase; Cyt b, cytochrome b. *Observed in proband and control hybrid cells.

that this substitution may not be significantly pathogenic. On the other hand, 14577 T/C causes an amino acid replacement (Table 1), which is conserved in at least human, bovine, mouse, and rat. Thus, we speculate that the 14577 T/C mutation is a major pathogenic mutation, if not the only mutation, of diabetes of the proband. However, because the 6 substitutions are in linkage disequilibrium, it is possible that the combinations with the other 5 substitutions may also contribute to the pathogenesis of diabetes of the proband.

We previously reported that >90% of heteroplasmy was needed to show a decrease in mitochondrial functions using a cybrid system (12). Chomyn et al. (13) reported similar data. Recent literature indicates that heteroplasmic threshold, if it impairs mitochondrial functions, is expected to be $\geq 90\%$ in cybrid cells and $\geq 85\%$ in muscle. So, we think that low heteroplasmic substitutions, which we could not detect by direct sequencing, are not likely to be the actual pathogenic mutations. Although we cannot completely rule out the existence of other very low heteroplasmic substitutions in the proband and the control cybrid cells, we think that the

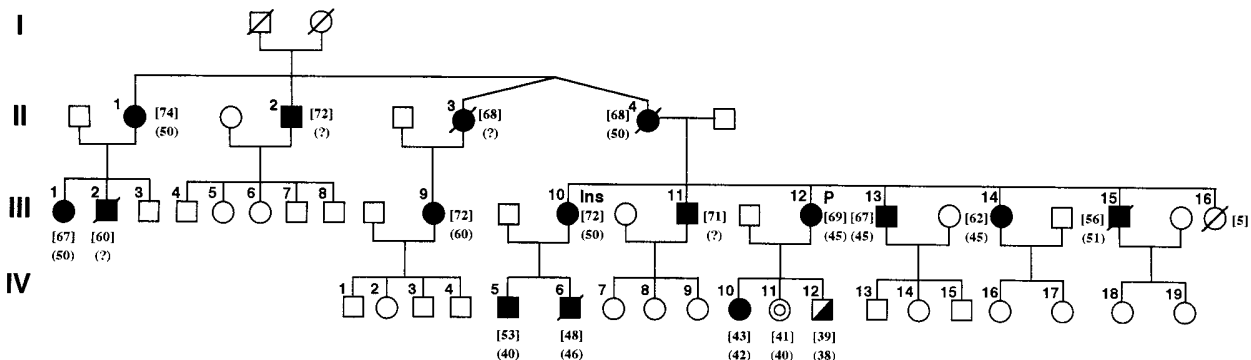


FIG. 2. Family pedigree. Subject III-12 was the proband (P). Symbols marked through by a slash indicate the subject is deceased. Subject III-10 was using insulin (Ins). Men are indicated by circles; women are indicated by squares. Diabetes is indicated by a closed symbol; impaired glucose tolerance is indicated by a half-closed symbol. The double circle indicates normal glucose tolerance. Numbers in parentheses and brackets indicate the age of diagnosis and the current age or the age of death, respectively. In this family, the mean known age of diagnosis is 48.4 years.

TABLE 2
Prevalences of mtDNA homoplasmic substitutions in Japanese subjects

Nucleotide substitutions	Control subjects	Type 2 diabetic subjects	<i>P</i>
3010 G/A	121 of 381 (31.7)	84 of 253 (33.2)	NS
3206 C/T	7 of 529 (1.32)	8 of 253 (3.16)	0.095
5178 C/A	146 of 381 (38.3)	102 of 253 (40.3)	NS
8414 C/T	119 of 381 (31.2)	84 of 253 (33.2)	NS
14577 T/C	0 of 529 (0)	3 of 253 (1.18)	0.0336
14979 T/C	6 of 529 (1.13)	6 of 253 (2.37)	NS
3010, 5178, and 8414	119 of 381 (31.2)*	84 of 253 (33.2)*	NS
All substitutions	0 of 529 (0)	3 of 253 (1.18)*	0.0336

Data are *n* (%), as determined by Fisher's exact test. Linkage disequilibrium was analyzed by χ^2 analysis. NS, not significant. **P* < 0.0001 for linkage disequilibrium.

absolute contribution of very low heteroplasmic substitutions to mitochondrial functions can be offset by the comparison between the 2 different cybrid clones. Therefore, we think it is reasonable to attribute the impairment in complex I activity of the proband to these substitutions, specifically to 14577 T/C mutation.

Swerdlow et al. (14) also reported the impairment in complex I activity in cybrid cells between ρ^0 human neuroblastoma cells and mtDNA derived from patients with Parkinson's disease. In addition, mutations at 14484 and 14459 in ND 6 have been reported in Leber hereditary optic neuropathy (LHON) (15). However, no family members of the present study had Parkinson's disease, LHON, or any other mitochondrial encephalomyopathies. Moreover, we did not observe any mitochondrial encephalomyopathies in 2 other diabetic patients associated with the 14577 T/C mutation. Therefore, we propose a new subgroup of type 2 diabetes associated with mtDNA 14577 T/C mutation. In general, phenotypes associated with mtDNA mutations show considerable variability. Although the mechanisms of the differences in phenotypes associated with mtDNA mutations are not fully understood, possible explanations may be due to the difference in organ-specific thresholds for energy demand, the difference in heteroplasmic rate in various organs, and/or the presence of additional nuclear gene mutation(s) (3).

In conclusion, the present study strongly indicates that the new mtDNA mutation at 14577 T/C may probably be a major pathogenic mutation in this family of maternally inherited type 2 diabetes. However, how mitochondrial impair-

ment leads to the development of type 2 diabetes is not completely clear at present.

RESEARCH DESIGN AND METHODS

The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all of the subjects before the study, and the study was approved by the Ethical Committee of Yamanashi Medical University.

Proband and family. We investigated a 69-year-old female patient with type 2 diabetes. Her current and maximal BMI were 24.3 and 30.25 kg/m², respectively. Glycemic control during the last 2 years was relatively good with HbA_{1c}, ranging from 6.0 to 6.9% by oral agents. Although the proband had 22 years of known duration of type 2 diabetes, she had no history of insulin treatment and no evidence of retinopathy or nephropathy and showed only mild neuropathy. The phenotype of the proband is different from that associated with 3243 mtDNA mutation (5). Figure 2 shows the family pedigree. There were 16 diabetic patients in this family with a mean known age of diagnosis at 48.4 years. The mode of transmission was a typical maternal inheritance. No member of this family showed clinical evidence of Parkinson's disease, optic atrophy, epilepsy, or encephalomyopathies.

Southern blot analysis and mtDNA investigations. Genomic DNAs were prepared from peripheral leukocytes, buccal mucosa, fibroblasts, and cybrid cells of the proband peripheral leukocytes of the relatives and from control cybrid cells by DnaQuick kit (Dainippon, Osaka, Japan). Approximately 15.6 kb mtDNA was obtained by long PCR using LA Taq (Takara, Kyoto, Japan). Genomic DNA (3 μ g) was digested by *Pvu* II (Takara) and separated by electrophoresis using an 0.8% agarose gel. DNA was transferred to a nylon membrane and hybridized with the probe. The entire mtDNA sequence was determined by direct sequencing. PCR was performed using a Takara PCR kit (Takara Shuzo, Kyoto, Japan), and the products were recovered using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The recovered templates were used for the second asymmetric PCR with a primer ratio of 1:50. The products of the asymmetric PCR were used as templates for sequencing using the Cycle Sequencing kit (Takara Shuzo). Oligonucleotides were designed as primers for sequencing to cover every 220 bp of each template. The A/G heteroplasmic mutation at 3243 was also detected by *Apa* I digestion after PCR with labeled primers (4). The vicinity of 14577 was amplified from genomic DNA of the proband by PCR with *Xho* I/*Eco* RI linker primers, and the products were digested with *Xho* I/*Eco* RI, ligated into pBluescript SK(-), and then transformed into XL-1 blue cells. A total of 93 clones were isolated and sequenced using T7 primer to verify the heteroplasmic rate at 14577 T/C.

Screening. The prevalence of mtDNA nucleotide substitutions were investigated in 252 unrelated patients with type 2 diabetes (253 patients including the proband) (mean age 55.7 \pm 1.8 years). Samples were obtained from patients in the order of their visits to the outpatient clinic of Yamanashi Medical University Hospital. We also recruited 381 healthy control subjects (mean age 42.5 \pm 1.5 years) with no family history of diabetes within fourth-degree relatives and with either a normal oral glucose tolerance test or normal HbA_{1c} and fasting blood glucose values. These subjects visited Isawa Kur Haus or Koseiren Health Center for routine medical check-ups. Genomic DNA was obtained as described above. Furthermore, collected genomic DNA samples of another 148 control subjects, based on the same criteria previously described, were kindly provided by Dr. T. Yanagawa (Nerima General Hospital, Tokyo). Thus, the total number of control subjects was 529 (mean age 48.2 \pm 1.8 years).

TABLE 3
Mitochondrial respiratory functions in cybrid cells

	Control cells	Proband cells
<i>n</i>	12	3
Complex I activity (nmol \cdot min ⁻¹ \cdot mg protein)	125 \pm 7.8	44.4 \pm 7.8
Complex IV activity (nmol \cdot min ⁻¹ \cdot mg protein)	117.9 \pm 17.5	97.5 \pm 14.9
O ₂ consumption (nmol O ₂ \cdot min ⁻¹ \cdot 10 ⁻⁷ cells)	28.1 \pm 8.1	10.8 \pm 5.7

Data are *n* or means \pm SD. Cybrid cells between ρ^0 HeLa cells and mtDNA from either 3 control subjects or the proband were obtained by cell fusion techniques.

Cybrid study. ρ^0 HeLa cells were prepared by treating the HeLa cells with ethidium bromide as previously reported (16). ρ^0 HeLa cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS) containing 0.15% NaHCO_3 , gentamicin (50 $\mu\text{g/ml}$), nystatin (100 U/ml), pyruvate (1 mmol/l), and uridine (20 $\mu\text{mol/l}$) (17). Exogenous mtDNAs were transferred to ρ^0 HeLa cells in the presence of 50% polyethylene glycol 1500 (Boehringer Mannheim, Mannheim, Germany) by using platelets obtained from the proband or 3 age-matched control subjects as the mtDNA donors (18). The fusion mixture was cultured in the DM 170 selection medium without glucose (Kyokuto Chemical, Tokyo) supplemented with 10% FBS containing 0.1% NaHCO_3 , gentamicin (50 $\mu\text{g/ml}$), and nystatin (100 U/ml) (17). Cybrid cells were cultured in the selection medium for 2–3 weeks after the fusion. Then, cybrid cells were grown in RPMI-1640 with 10% FBS containing 0.15% NaHCO_3 , gentamicin (50 $\mu\text{g/ml}$), and nystatin (100 U/ml) for 2–3 weeks and were cloned by the cylinder method. Activities of mitochondrial respiratory chain complexes I (19) and IV (20) and the rate of O_2 consumption (16) were measured as described.

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